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Coagulation factor VIIa, a circulating serine protease, binds to its cofactor, tissue factor (TF), to trigger the blood clotting cascade. TF is a glycoprotein present on the surface of a variety of cell types found outside of the vasculature, and various types of cancer cells. TF:VIIa complex formation ultimately leads to fibrin polymerization and platelet activation, in both hemostasis and thrombotic disease. Complex formation has also been shown to activate certain signaling cascades, altering cellular properties such as adhesion, migration, and apoptotic potential. Many types of cancer cells have been shown to express high levels of TF. Blockage with anti-TF antibodies has demonstrated that metastasis depends on the presence of catalytically competent TF:VIIa complexes. Such cellular alterations, high morbidity associated with thrombosis, and the privileged position of VIIa in the coagulation cascade, make inhibition of TF:VIIa an important issue in cancer therapy. We have demonstrated that antithrombin (AT) can inhibit TF:VIIa, when in the presence of heparin, and AT:VIIa complexes have been detected in plasma, suggesting that VIIa is inhibited by AT *in vivo*. We have also shown that AT can reversibly inhibit factor VIIa *in vitro* and in plasma, and are currently beginning studies in breast cancer and endothelial cells.

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The Effects of Antithrombin and Tissue Factor Pathway Inhibitor on the Tissue Factor:Factor VIIa Complex on the Surface of Breast Cancer Cells: Implications on Blood Coagulation, Gene Expression, Cell Adhesion and Metastasis.

Annual Summary Report April 2003
Francesca Antonaci (Award Number DAMD17-02-1-0410)

Introduction: Coagulation factor VIIa (VIIa) is a circulating serine protease which, upon binding to its cofactor, tissue factor (TF), is responsible for triggering the blood clotting cascade in both normal hemostasis and in thrombotic disease (reviewed in 1). TF is a type I integral membrane protein present on the surface of a variety of cell types found outside of the vasculature, and on various types of cancer cells (2-5). The TF:VIIa complex catalyzes the maturation of factors IX and X to the activated factors IXa and Xa by limited proteolysis. Activation of these coagulation factors ultimately leads to fibrin polymerization and platelet activation, thereby plugging the vessel wall at the site of injury, or forming a clot within the vasculature, in the case of thrombotic disease and cancer. Binding of VIIa to TF has also been shown to activate certain signaling cascades, such as MAP kinase pathways, and to increase intracellular calcium levels, thus altering cellular properties such as adhesion, migration, and potential for apoptosis (reviewed in 6, 7). Many types of cancer cells have been shown to express high levels of tissue factor, and blockage of VIIa binding by use of anti-TF antibodies has demonstrated that metastasis, in these cells, is dependent on the presence of catalytically competent TF:VIIa complexes (8).

Alteration of cellular properties due to signaling through TF, the high morbidity associated with thrombosis in various disease states, and the privileged position of VIIa in the coagulation cascade, make inhibition of the TF:VIIa complex a very important issue which requires further study. There is currently only one human protein that is widely accepted as an inhibitor of the TF pathway, namely, tissue factor pathway inhibitor (TFPI) (reviewed in 9). TFPI, however, can only inhibit TF:VIIa after binding to an activated Xa molecule, and the active inhibitor is not found in high levels in plasma, leading to the notion that there may be more than one physiological inhibitor of the TF pathway. Some laboratories were able to show that antithrombin (AT), a serine protease inhibitor (serpin) that is most well known as an inhibitor of thrombin (factor IIa) and factor Xa, can also inhibit TF:VIIa, when in the presence of the glycosaminoglycan (GAG), heparin (10-12). The presence of AT:VIIa complexes has been demonstrated in plasma; the concentration of these complexes is higher than the concentration of free VIIa, demonstrating that VIIa can be inhibited by AT (unpublished results). I have recently demonstrated that the inhibition of TF:VIIa by AT/heparin has a rate constant of approximately 2.5×10^4 $M^{-1}s^{-1}$. The rate constant for TFPI inhibition of TF:VIIa is much higher (1.6×10^7 $M^{-1}s^{-1}$) (13), but the plasma concentration of AT is 2μM, whereas active TFPI is present in very low levels in the vasculature under normal conditions (14), suggesting that AT may be at least as important as a physiological inhibitor of TF:VIIa as TFPI. Although AT requires heparin for efficient inhibition of TF:VIIa, I postulate that glycosaminoglycans, such as heparan sulfate, present on the endothelium of vessel walls may be able to activate antithrombin similarly to heparin, thereby leading to inactivation of VIIa.

Results: During the past year, I have focused my efforts on gathering evidence to support the hypothesis that AT is, in fact, an important physiological inhibitor of VIIa; in more recent experiments, I have concentrated on demonstrating that AT can reversibly inhibit VIIa in the absence of TF, and whether this inhibition occurs by a competitive mechanism. I have shown that AT can inhibit VIIa in the absence of TF, but that heparin is required to activate AT. I used a small chromogenic substrate to assay for VIIa activity. When plasma levels of AT were used

(2μM), along with heparin, 70% inhibition can be attained after 20 minutes at 37°C. Upon 10 fold dilution of the above reaction full recovery of VIIa activity is seen, indicating a reversible mechanism for inhibition in the absence of TF. Because results with a small substrate cannot fully mimic VIIa activity on X activation *in vivo*, I next tested the effects of dilution of AT reactions in clotting assays. Clotting assays were performed by adding pooled normal plasma to reactions with VIIa, AT, heparin, and TF. The reactions were pre-incubated for 20 minutes at 37°C, and then diluted 1000 fold and reincubated for 20 minutes at 37°C to allow for dissociation of antithrombin from VIIa. I found that clotting time is severely delayed when AT and VIIa are incubated in the presence of TF, and that dilution of these reactions does not allow for full recovery of activity. When TF is not present, AT is able to delay the clotting time, but after dilution, full recovery of activity can be achieved, further demonstrating that there may be an additional novel mechanism for AT inhibition of VIIa. I have used Western blotting to determine what levels of covalent complex formation is seen using various reaction conditions. Covalent complexes are identified as an up-shifted band visible after SDS-PAGE electrophoresis and blotting with an anti-VII or anti-antithrombin antibody. High levels of these complexes are seen after incubation of VIIa with AT, heparin, and soluble TF. Very low levels of the complex are seen when TF is absent, indicating that most inhibition seen in *in vitro* assays is due to an interaction that does not result in covalent complex formation. Complex formation is very dependent on the presence of heparin, but GAGs on cell surfaces of the vessel walls may be able to substitute for heparin in activating AT. To determine by which type of mechanism this inhibition is occuring, I began studies to determine V_{MAX} and K_M, but results thus far have been complicated, and may indicate a mixed mechanism in which there is a low affinity binding that leads to reversible inhibition by a competitive mechanism (through active site interactions), along with a higher affinity binding that leads to irreversible complex formation. More experiments will need to be completed in order to verify these results.

I also worked on probing for AT:VIIa complexes in plasma using immunoprecipitation and Western blotting methods. An anti-VII antibody was coupled to an Affigel matrix using the manufacturer's protocol, and various types of plasma were then incubated with the antibody-bead slurry for three hours in order to bind VII, VIIa, and its complexes. The beads are then washed, and VII/VIIa is eluted using SDS sample buffer. Samples are separated by SDS-PAGE electrophoresis and blotted with either a biotinylated anti-VII antibody or a polyclonal anti-antithrombin antibody. It is important to mention that this antibody is successful at co-immunoprecipitating full length AT, as well as AT in complex with VIIa, providing further evidence that VIIa can interact with AT in a non-covalent manner for long periods of time. Complexes are seen in plasma alone, but are difficult to detect due to lack of sensitivity of the Western blot. When 100nM VIIa is added back to the plasma, large amounts of AT and AT:VIIa complexes precipitate with the antibody. Heparin and soluble TF enhance the precipitation several fold, but are not required for complex formation at these high concentrations. It would be of interest to add cells to the assay which express GAGs on their surfaces, but not TF, to determine whether they can enhance complex formation, as heparin does.

During the months since my preliminary examination I have worked at reconfirming that vitronectin, an abundant plasma protein, enhances AT inhibition of VIIa. I eventually concluded that my preliminary data was incorrect, and that vitronectin does not enhance this inhibition. I also spent some time showing that ELISA data from the lab were accurate. Plasma samples which showed low levels of VIIa:AT complexes in ELISAs, when compared with plasma samples with high levels of VIIa:AT showed lower intensity banding on Western blot (shown by coimmunoprecipiation). Finally, I decided to try to reconfirm my results that AT inhibition of VIIa in the absence of TF is reversible, but have had technical difficulties due to heparin interfering with VIIa activity. Heparin has not previously been documented to inhibit VIIa

activity, and has not been shown to even bind to VIIa. We are currently looking into methods to clear up this problem. I have also begun competition studies to show that high levels of VIIa can compete with thrombin for AT binding, and that this binding is reversible by increasing incubation times with thrombin, but the heparin I was using has proved unsuitable for this type of experiment. I am awaiting heparin pentasaccharide in order to more clearly be able to identify competition between these proteins and to show a reversible mechanism of inhibition.

Key Accomplishments:

- Demonstrated that AT can inhibit TF:VIIa *in vitro* when heparin is present;
- VIIa:AT complexes are present in plasma at higher concentrations than free VIIa indicating that AT inhibits VIIa *in vivo* as well (developed co-immunoprecipitation protocol to reconfirm lab ELISA results);
- Showed that AT may therefore be an important physiological inhibitor of TF:VIIa;
- Demonstrated that AT can also inhibit VIIa activity in the absence of TF;
- Gathered evidence to show that this inhibition seems to be largely reversible *in vitro* and in plasma;
- Used new co-immunoprecipitation protocol to show that VIIa:AT complex formation is largely dependent on the presence of an activating factor, such as heparin or TF;
- Showed that anti-VII antibody co-immunoprecipitates uncomplexed AT from plasma, providing evidence for a non-covalent interaction of AT and VIIa in plasma;
- Early kinetic studies suggest that this inhibition may occur by a mixed mechanism.

Problems: I changed laboratories in 2002 from Dr Shapiro's laboratory to Dr Morrissey's laboratory. That has caused considerable changes in my plans and also a bit of disorganization. Last year I was require to submit a revised proposal around the time I joined the new lab. Because of this I did not file my first annual summary report until November of 2003. I did not realize at the time that I was in default because I believed that my new proposal was all I could provide at the time the annual summary report was due, because I had not yet started work in the new laboratory. Because of this the 2004 summary report does not contain as much new material as I would like, as it covers only four months of work rather than an entire year. In addition I have had a large amount of technical difficulties with the enzymatic assays, and so do not yet have any cancer data ready, but can report this as it comes.

Reportable Outcomes:

Presentations:

- Preliminary Examination, September 2003; passed with a recommendation of "outstanding;"
- Poster presentation at Medical Scholars Program fall retreat; title: Determining the Nature of Inhibition of VIIa by AT: A Possible Novel Mechanism for Maintaining Hemostasis

- Poster presentation at Department of Biochemistry recruiting weekend; title: Determining the Nature of Inhibition of VIIa by AT: A Possible Novel Mechanism for Maintaining Hemostasis (new poster with new data despite same title)
- Departmental Seminar presentation, April 2004; title: Antithrombin Inhibition of Coagulation Factor VIIa.

Conferences Attended:

- International Society of Thrombosis and Haemostasis Meeting in Birmingham, England, July 2003;
- Second International Conference on Thrombosis and Hemostasis Issues in Cancer, Bergamo, Italy, September 2003.

Conclusions and Future Directions:

In the near future I plan to repeat competition studies using factor Xa instead of thrombin, and to obtain pentasaccharide in order to more cleanly determine whether VIIa is able to compete with thrombin for AT, and to show whether this interaction is reversible by increasing incubation times with thrombin. I plan to investigate problems with the lot of VIIa I am using; one technical problem I am having with repeating my reversibility data in the amidolytic activity assay system is that activity of VIIa alone has gone down by 50% since I first did these experiments one year ago. The activities are so low that it is hard to document whether VIIa is regaining activity after dilution. If able to repeat these studies, I will then attempt to show reversibility using a macromolecular substrate for VIIa, factor X, to compare with the results using small peptidyl substrates.

Although the rate constant for inhibition of TF:VIIa by AT/heparin is quite low, it still may be significant enough to play some role physiologically. TFPI levels are low in plasma, indicating that there may be another circulating inhibitor of VIIa. There are normally small amounts of active VIIa in blood, but the question then remains why blood clotting does not normally occur spontaneously, even though there have been reports of TF circulating in plasma (reviewed in 15). We hypothesize that VIIa must be present in blood in a reversibly inhibited form, perhaps bound to antithrombin. Further studies must be completed on the type of inhibition mechanism AT uses in the absence of TF. It is also necessary to study the effects of endothelial and cancer cells with GAGs present on their surfaces on AT inhibition of VIIa activity in the presence and absence of TF. It would be interesting to see what effects AT has on TF signaling by assaying for changes in cell adhesion, metastatic potential, apoptosis, and other parameters. I also hypothesize that AT inhibition of TF/VIIa might be important in blocking the propagation of blood coagulation, and that, since the rate constant is so low, it might be in place in order to allow coagulation to initiate and propagate normally, but that it then acts as a brake to ensure that wide-spread coagulation does not occur. These hypotheses provide novel roles for AT in inhibition of blood proteases, and support the role of heparin as an important anticoagulant.

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